

Laboratory techniques

Laboratory techniques in the investigation of fungal infections

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Introduction

Fungal infections, mainly in the form of vaginal candidosis, are common in patients attending genitourinary medicine clinics. However, infections affecting the AIDS patient extend this range of fungal disease considerably. The mycoses in AIDS patients include some of the commoner and more important complications of this disease such as oropharyngeal candidosis and cryptococcosis. Rarer examples include extensive dermatophytosis, histoplasmosis and penicilliosis (table). The clinical diagnosis of most of these diseases usually needs to be confirmed by appropriate laboratory tests.^{1,2} This is a particular problem in the AIDS patient where classical symptoms such as headache in meningitis may be minimal and clinical signs altered. Dermatophytosis, for instance, although not apparently commoner in the AIDS group may present with strikingly different clinical features compared with the otherwise healthy patient.³ Some of the rarer fungal infections which may also present in this group, such as histoplasmosis, sporotrichosis and penicilliosis, are not endemic in Europe and their recognition may be delayed because of unfamiliarity.

Candidosis

Infections due to yeasts belonging to the genus *Candida* are common occurrences in genitourinary medicine as in other branches of medicine. The infections range from superficial disease such as oral or vaginal infection to deep or systemic infections. *Candida albicans* or related species are commensals in the mouth, gastrointestinal tract and the vagina. The carriage rates vary considerably in different studies with 16-45% of the normal population being carriers. Infection is accompanied by a change in the status of the organism from saprophyte to parasite. In some, but not all, instances this is accompanied by a change in

morphology from yeast to hyphal form. The commonest of the pathogenic *Candida* species is *C. albicans* but *C. tropicalis*, *C. krusei* and *C. glabrata* amongst others can all cause human infections. This raises two important points relevant to diagnosis. Firstly it is important to distinguish between commensal *Candida* and those forms causing infection. Secondly it may be important to identify the organisms isolated to species level because their responses to therapy may differ.

The simplest method of recognition of *Candida* in smears or swabs from the vagina or mouth is by direct microscopy.⁴ Smears can be examined mounted either in 5-10% potassium hydroxide or stained with the standard Gram procedure. The organisms usually appear as yeasts with regular hyphae and in some areas elongated chains of yeast-like organisms, pseudohyphae. Other stains which have been used to demonstrate *Candida* include Wright's stain or periodic acid Schiff although the latter is quite time consuming. Calcofluor white (Polysciences Inc.) involves using an optical whitening agent, generally with Evan's blue as counterstain, to highlight cells, although it is necessary to examine the preparation with a fluorescence microscope.¹

Sampling for culture depends on the use of standard cotton tipped swabs.¹ These are best moistened in the transport medium prior to sampling. As with most microbial specimens delay in sending the material to the laboratory will result in results which are difficult to interpret as overgrowth with more rapidly growing bacteria may occur. In the oropharynx mouthwash samples may provide higher yields. The usual medium for isolation is Sabouraud's glucose peptone medium (1% mycological peptone, 4% glucose and 1.5% agar). This normally contains antibacterial antibiotics such as chloramphenicol (0.005%) or penicillin (20 mg/l)/streptomycin (40 mg/l) to suppress bacterial growth. Where mixed infections are suspected dual plates will be needed. There are some commercially available kit systems specifically designed for isolation of yeasts from the vagina. Generally these are more expensive but include dip slides. In addition a latex test for detecting *Candida* antigen in the vagina has been developed although its success rate does not seem to exceed that using direct microscopy in experienced hands.⁵ *Candida* species are not generally fastidious and will grow on other non-specific media such as blood agar. *Candida* species as other yeasts are usually ready for identification within 48 hours. Specific culture methods are not usually

Table Mycoses in the AIDS patient

Superficial mycoses:
Dermatophytosis
Candidosis (oropharyngeal, vaginal)
Diseases due to <i>Pityrosporum</i> yeasts (seborrhoeic dermatitis)
Rare: alternariosis, white piedra
Subcutaneous mycoses:
Sporotrichosis
Systemic mycoses:
Cryptococcosis
(Aspergillosis)
Histoplasmosis
Coccidioidomycosis
Rare: blastomycosis, disseminated infection due to <i>Penicillium marneffei</i> (penicilliosis)

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necessary for the isolation of *Candida* from superficial sites but if the organisms are being sought in blood cultures there is some evidence that lysis centrifugation is a preferred method.⁶

The identification of *Candida* species is then subsequently based on a simple test the germ tube test whereby the yeasts are incubated for 2 hours at 37°C in non-immune human serum.⁴ *Candida albicans* forms germ tubes, small outgrowths from the yeast cells under these conditions. This is a simple distinguishing test to separate *C. albicans* from the other species. There are occasional false positives particularly where *C. tropicalis* is present and if the test is incubated for longer than 2 hours. There are more detailed investigations available to the laboratory,⁷ the simplest of which the API system is based on the utilisation of sugars (for example API Laboratory Products Ltd); by presenting a battery of sugars in a strip culture it is possible to speciate most yeasts. More complex approaches such as culture morphology on different agars and assimilation reactions or restriction fragment analysis are used only in specialised laboratories;⁸ however, they have proved useful in tracking outbreaks of infection.⁹⁻¹⁰ Other approaches to recognition of *Candida* species such as the detection of amplified DNA fragments using the polymerase chain reaction have only recently been applied to the recognition of yeasts.¹¹

Superficial infections are usually caused by *C. albicans* although other species, such as *C. glabrata*, may be involved in vaginal candidosis. Topical antifungals work well against most species. However identification of the species may be helpful if there is doubt about the role of the organism isolated in causing disease or in chronic treatment unresponsive infections because the sensitivity of different *Candida* species to different orally active azole antifungals may vary.¹²⁻¹³ For instance *C. tropicalis* is often less sensitive to ketoconazole; *C. glabrata* and *C. krusei* are less sensitive to fluconazole.

There is no need to use serodiagnosis in superficial candidosis although there are a number of serodiagnostic systems in use for systemic infections, most of which have important limitations.¹⁴⁻¹⁵

Interpretation of laboratory results. Generally the best method of ensuring that the organisms isolated are pathogenic is by relating the culture results to the presence or absence of clinical disease. Clues from laboratory investigation, which may indicate the presence of infection rather than colonization, include the presence of hyphae amongst yeasts seen in microscopy of smears and the isolation of large numbers of organisms.

Other superficial infections

The commonest of the other superficial fungal infections is dermatophytosis, although pityriasis versicolor may also develop in some patients. The incidence of either disease is not increased in AIDS patients although dermatophytosis may be difficult to recognise because lesions appear atypical.¹⁶ The normal labo-

ratory diagnosis is simple and involves the use of direct microscopy and culture.¹⁷ Samples are generally scrapings taken with a scalpel from the edge rather than the centre of lesions. The technique for microscopy is the same as that used for candidosis (5–10% potassium hydroxide). Dermatophyte cultures generally grow best at room temperature although it may take at least 7–10 days before the isolates are sufficiently well grown to identify. Scrapings for pityriasis versicolor are taken in the same way. It is not necessary to culture the organisms which are fastidious lipophilic yeasts as their microscopic appearances are typical. There is usually a mixture of round yeast forms and short stubby hyphae. A stain using a 1:1 mixture of Parker's Quink ink and potassium hydroxide (10%) will highlight these fungal elements very clearly and provides a rapid method of screening scrapings.

Cryptococcosis

Cryptococcosis is a systemic yeast infection caused by an encapsulated yeast, *Cryptococcus neoformans*. This is present in the environment particularly in areas contaminated with pigeon excreta. The route of infection is via the lungs but the commonest clinical manifestation is meningitis or another feature indicating dissemination eg skin or bone lesions. While it is thought that many individuals are sensitised, but not infected, after exposure, in the severely immunocompromised patient particularly those with T lymphocyte defects there is a higher risk of the emergence of clinical infection. The commonest underlying disease in cryptococcosis is AIDS. In the UK it causes disease in about 3% of AIDS patients, whereas in Zaire the incidence of infection is higher—about 12%.¹⁸

Cryptococcosis in AIDS patients differs to some degree to that seen in other groups. For instance there is a higher frequency of cryptococcaemia and the symptoms may be comparatively mild.¹⁹ The principles of diagnosis however are the same whatever the patient's background.

Cryptococcus neoformans, which is an encapsulated organism, can be demonstrated by direct microscopy.¹ The usual techniques used for demonstrating the organisms in smears or CSF are the India ink or Nigrosin stains. Both techniques rely on the fact that the capsule will displace an opaque medium such as India ink particles and the clear halo around a single cell will represent the diagnostic capsule of the infecting organism. The method of carrying out the test is simple. A drop of material such as cerebrospinal fluid is mixed with an equal volume of India ink on a glass slide, covered with a coverslip and examined. Some macrophages can produce a similar phenomenon so the slide should be carefully scanned for budding yeasts; this is less likely to happen if nigrosin is used as the stain. Some cryptococci are only weakly encapsulated and these may be difficult to separate from lymphocytes. This may be more commonly seen in isolates from AIDS patients.²⁰ Once again the presence of buds is diagnostic.

Cryptococcus neoformans is normally easy to isolate on Sabouraud's agar from clinical material.² It grows best at 30–31°C and may appear slowly (up to 10 days) on primary isolation producing white to yellow colonies which may appear mucoid. *C. neoformans* produces urease and this forms the basis of a simple diagnostic test. It also turns brown on *Guizotia abyssinica* seed agar because of melanin production. Sugar assimilation can also be used for identification using the API system. The cultural identification of *C. neoformans* is therefore specific and comparatively simple. Culture not only confirms the diagnosis it also provides some insight into prognosis. The rapidity of clearance of the organism from the CSF, for instance, is a good reflection of recovery in AIDS patients. Other sites which may harbour *C. neoformans* include skin, prostate²¹ and bone and in AIDS patients about 20–30% have positive blood cultures, an unusual finding in the non-AIDS group.¹⁹

A slide agglutination test for the presence of capsular antigen in CSF or serum using antibody-coated latex particles (Immutech Ltd) is useful and can provide a diagnosis in about 30 minutes.²² False positive reactions may be seen unless the CSF or serum is strongly heated or treated with dithiothreitol because of the presence of antiglobulins. Monoclonal antibodies to cryptococcal capsular antigen have been prepared and these can be used in a different system.^{23, 24} For instance a new antigen detection method using an ELISA system (Meridien Diagnostics) is also available. Both systems detect the presence of capsular polysaccharide antigen and the titres give some indication of the extent of infection and indirectly of the prognosis. AIDS patients usually have antigen in both serum and CSF and this only declines slowly with therapy. The detection of antigen has advantages because the tests are simple and very rapid. There is some cross reactivity with one organism, *Trichosporon*, usually a cause of systemic infection in the neutropenic patient, but otherwise this is not a problem.²⁵ By contrast antibody detection has little role to play in the diagnosis of cryptococcosis. Tests are only positive in about 20% of cases.

Cryptococcus neoformans can also be distinguished in biopsy material. The yeasts are generally pleomorphic and may range in size from 4–15 μ m in diameter. They stain with specific fungal stains such as methenamine silver (Grocott modification) or periodic acid Schiff (PAS).²⁶ In addition the mucicarmine stain will specifically highlight the cryptococcal capsule even in organisms which have small capsules. This is therefore a specific stain for *Cryptococcus*. The use of CT of the head may highlight changes such as cortical atrophy or ventricular enlargement but these are not specific.²⁷

Histoplasmosis

Histoplasmosis is a systemic fungal infection seen in AIDS patients as well as those without underlying disease. It is endemic in areas of the USA, South America, Africa and Asia. Expo-

sure in communities in these areas may be high with up to 70% of the population having positive histoplasmin skin tests indicating sensitisation. The route of infection by the causative fungus *Histoplasma capsulatum* is via the lungs and the infection may resolve at this point or remain localised to the lung or disseminate from there to other sites. AIDS patients generally present with systemic infections.²⁸ These involve the reticuloendothelial system and patients present with fever and lassitude together with hepatosplenomegaly and skin or mucosal papules or ulcers.

The diagnosis of histoplasmosis is not difficult to confirm by laboratory tests.¹ The organisms are very small, 2–4 μ m in diameter, and cannot be seen with direct microscopy unless highlighted with a Giemsa stain in white cells or bone marrow. Sources of material for culture include blood, bone marrow, skin lesions or sputum. The organisms grow well in Sabouraud's agar culture at 25–30°C, although to confirm the diagnosis it is necessary to convert organisms from mycelial to the yeast phase on enriched media such as Brain Heart infusion agar at 37°C or identify specific antigen eluted from the culture and probed with a specific antiserum in an immunodiffusion plate (exoantigen assay).²⁹ The main serological tests are the complement fixation assay and an immunodiffusion test.²² However, a new RIA test for detection of galactomannan antigen is available in some centres.³⁰ A histoplasmin skin test, similar to a Mantoux test, is available, although it is only useful for investigating the epidemiology of infection. A positive histoplasmin test merely indicates exposure to the organism and many patients with disseminated infection appear to be anergic and have negative skin tests.

Other systemic pathogens

The other systemic fungal pathogens are comparatively rare, even in AIDS patients. In areas endemic for coccidioidomycosis (South western semidesert areas of the USA and similar climatic zones in South America) this infection can be seen. It may present with pulmonary or disseminated lesions. Smears will reveal the causative organisms which develop into large rounded structures or spherules which contain endospores. *Coccidioides immitis* grows readily in culture although it is a well recognised cause of laboratory acquired infection and the laboratory should be warned if this infection is suspected.³¹

Unusual infections will occur in any group of immunocompromised patients and the identification of rare pathogens usually requires the help of a reference laboratory. Where a delay is likely it may be necessary to initiate treatment with a broad spectrum antifungal agent if the patient's condition is deteriorating. This can be changed if necessary later, although most fungal pathogens seen in AIDS patients will respond to amphotericin B.

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